Mycobacterium xenopi and related organisms isolated from stream waters in Finland and description of Mycobacterium botniense sp. nov.

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Three scotochromogenic Mycobacterium xenopi-like organisms were isolated from stream waters in Finland. These strains grew at 36–50 °C but not at 30 °C. One of the three strains was fully compatible with the M. xenopi type strain according to GLC-MS, biochemical tests, and 16S rDNA and 16S–23S rDNA internal transcribed spacer (ITS) sequencing. Two of the strains closely resembled M. xenopi in lipid analyses and biochemical tests, but analysis by GLC-MS verified the presence of two new marker fatty acids (2,4,6,x-tetramethyl-eicosanoic acid and 2,4,6,x,x-pentamethyl-docosanoic acid). The 16S rDNA and ITS region sequences of these two strains differed from those of M. xenopi and other previously described mycobacterial sequences. Therefore, the strains are regarded as new species of slow-growing mycobacteria, for which the name Mycobacterium botniense sp. nov. is proposed. The chemical, physical and microbiological quality of the water reservoirs of M. xenopi and M. botniense are described. As far as is known, this is the first time that M. xenopi has been isolated from natural waters. The strains of M. botniense sp. nov. (E347T and E43) have been deposited in the ATCC as strains 700701T and 700702, respectively.

Keywords: Mycobacterium xenopi, Mycobacterium botniense sp. nov., fatty acids, 16S rDNA sequence, ITS sequence

INTRODUCTION

In a project established to evaluate natural reservoirs of environmental mycobacteria, a series of water samples from Finnish streams was examined (Iivanainen et al., 1993). Mycobacterial isolates were analysed for the whole-cell lipid composition by GLC and classified according to their GLC profiles and appropriate biochemical and growth characteristics. The present study is a polyphasic taxonomic characterization of Mycobacterium xenopi and related mycobacterial strains isolated from these natural waters. Among the 757 isolates analysed, three strains were tentatively identified as M. xenopi. Further analyses indicated that two of the three isolates formed a clear cluster, distinct from M. xenopi sensu stricto. It is proposed that they represent a previously unidentified species, for which the name Mycobacterium botniense sp. nov. is suggested.

METHODS

Bacterial strains. M. xenopi strains present in the strain collection of the Department of Clinical Microbiology, Kuopio University Hospital, Finland were reanalysed for lipid profiles, in order to evaluate the stability of fatty acid and mycolic acid cleavage product (MACP) profiles among M. xenopi. These strains included M. xenopi ATCC 19250T and ATCC 19971, 25 clinical isolates (27, 69, M116, 150, 1244, 1411, La 2593, 9533, 13082, 17598, 21074, 22145, 22259, 30476, 31631, 31686, 34279, 32746, 36276, 36342, 41334, 45979, 55507, 55508, 59389) and one isolate from a pig (4264). Three environmental mycobacterial strains (E43, E341 and E347T) were included in the study. They originated...
Two environmental strains (E43 and E347) were cultured on Middlebrook 7H11 agar supplemented with Middlebrook OADC enrichment (Difco) at 42 °C for 6–8 weeks. Fatty acid methyl esters and alcohols were prepared by acid methanolation, and analysed and identified as reported previously in detail (Torkko et al., 1998).

Environmental analyses. Characteristics of the drainage areas, and chemical, physical and microbiological quality of the water reservoirs in which \( \textit{M. botniense} \) sp. nov. and \( \textit{M. xenopi} \) were found were analysed as described previously in detail (Iivanainen et al., 1993).

RESULTS

Lipid analyses

The fatty acid patterns of all 26 \( \textit{M. xenopi} \) strains isolated from clinical or veterinary sources and the ATCC strains consisted of saturated and unsaturated straight-chain fatty acids as well as 10-methyl-octadecanoic acid (tuberculostearic acid). The known prominent alcohol markers of \( \textit{M. xenopi} \), 2-docosanol and 2-eicosanol (Alugupalli & Larsson, 1992), were detected, in addition to hexacosanoic acid (26:0) (Table 1). Dodecanoic acid (12:0) was also found to be a significant marker present in all strains (except strain 41334), in which it was present at a mean relative amount of 7.7\%.

The three environmental isolates had GLC fatty acid and alcohol profiles very similar to that of \( \textit{M. xenopi} \). However, two of them (E43 and E347), although otherwise similar, lacked dodecanoic acid.

GLC-MS analysis of the environmental strains E43 and E347 highlighted two further differences. Firstly, an additional compound was found to co-elute with the peak of 2-docosanol. Secondly, 2-tetracosanol, a minor marker of \( \textit{M. xenopi} \), was replaced by another compound which had an approximately similar retention time. These two compounds were found to be additional fatty acid methyl esters. They eluted between 20:0 and 22:0, and between 22:0 and 24:0, indicating that the main carbon skeletons of the compounds contained 20 and 22 carbons, respectively. The first of the peaks contained 24 carbons and the latter 27 carbons, resulting in mass peaks at \( m/z \) 382 and 424, which also indicates the presence of four and five methyl branches, respectively. The compounds contained no hydroxyl groups on the basis of trimethylsilyl derivatization (Torkko et al., 1998), nor did they have any double bonds. The mass spectra of the compounds showed ions at \( m/z \) 88 \([\text{CH}_2\text{O} \cdot \text{C} (\text{OH})]^{+}\), \( m/z \) 101 \([\text{CH}_3\text{O} \cdot \text{CO} \cdot \text{CH} (\text{CH}_3) \cdot \text{CH}_2]^{+}\) (base peak), \( m/z \) 129 and \( m/z \) 171, which confirmed the locations of three methyl groups at C-2, C-4 and C-6. The positions of the other
addition, the 16S rDNA of two randomly selected strains (E43, E341 and E347). The complete 16S rDNA of the three environmental isolates was sequenced. In addition, the 16S rDNA of two randomly selected clinical M. xenopi isolates (9533 and 45979), the clinical M. xenopi isolate lacking dodecanoic acid (41334), and the porcine isolate (4264) was partially sequenced. The partial sequence covered the region corresponding to Escherichia coli positions 30–900, which includes two of the most hypervariable regions. All of these 16S rDNA sequences were compared with that of the M. xenopi type strain ATCC 19250T (Rogall et al., 1990). Compared with the M. xenopi type strain, single nucleotide differences were detected in the variable regions of the 16S rDNA in porcine and clinical strains, and in one environmental isolate (E341). Strain 41334 had a difference of two nucleotides when compared with the type strain. The other two environmental isolates (E43 and E347) were identical. Their 16S rDNA sequence differed from all sequences

**Table 1. Fatty acid and fatty alcohol markers (mean and range of total peak area), useful for the classification of M. xenopi and M. botniense sp. nov.**

Fatty acid and alcohol markers: 1, dodecanoic acid; 2, 2-eicosanol; 3, 2,4,6,x-tetramethyl-eicosanoic acid; 4, 2-docosanol; 5, 2,4,6,x-x-pentamethyl-docosanoic acid; 6, tetracosanoic acid; 7, hexacosanoic acid.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Marker</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. xenopi ATCC strains (n = 2)</td>
<td>60 (19-81)</td>
<td>16 (12-20)</td>
<td>0</td>
<td>11 (10-12)</td>
<td>0</td>
<td>22 (19-22)</td>
<td>10 (8-13)</td>
<td></td>
</tr>
<tr>
<td>M. xenopi clinical strains (n = 2)*</td>
<td>76 (60-121)</td>
<td>20 (16-39)</td>
<td>0</td>
<td>11 (6-13)</td>
<td>0</td>
<td>18 (10-39)</td>
<td>11 (7-21)</td>
<td></td>
</tr>
<tr>
<td>M. xenopi porcine isolate (n = 1)</td>
<td>70</td>
<td>1.6</td>
<td>0</td>
<td>9.9</td>
<td>0</td>
<td>1.1</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td>M. xenopi environmental isolate E341</td>
<td>79</td>
<td>1.8</td>
<td>0</td>
<td>10.9</td>
<td>0</td>
<td>1.7</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>M. botniense environmental isolates E43, E347†</td>
<td>0</td>
<td>31 (29-33)</td>
<td>16 (15-170)†</td>
<td>3 (3-12)†</td>
<td>18 (1-18)</td>
<td>15 (11-19)</td>
<td>79 (59-81)</td>
<td></td>
</tr>
</tbody>
</table>

*One strain totally lacks dodecanoic acid.
†Amounts estimated from GLC-MS. The peak of 2,4,6,x-tetramethyl-eicosanoic acid has approximately the same retention time as 2-docosanol in GLC.

methyl branches were not determined. Accordingly, the first of the compounds was identified as 2,4,6,x-tetramethyl-eicosanoic acid (2,4,6,x-tetramethyl-20:0) and the latter as 2,4,6,x,x-pentamethyl-docosanoic acid (2,4,6,x,x-pentamethyl-22:0).

The HPLC mycolic acid profiles of the strains E43 and E347† were very similar to the known profile of M. xenopi (Tortoli & Bartoloni, 1996). They shared the same peaks, although the peaks had slightly different relative heights.

**16S rDNA sequencing and phylogenetic analysis**

The complete 16S rDNA of the three environmental strains (E43, E341 and E347†) was sequenced. In addition, the 16S rDNA of two randomly selected
available in the EMBL database. A similarity search indicated that this sequence was closest to that of the M. xenopi type strain, although there were 22 nucleotide differences detectable. A phylogenetic tree was constructed to show the location of the investigated isolate among slow-growing mycobacteria (Fig. 1).

**ITS sequencing of environmental isolates**

According to the ITS sequence analysis, E341 was identical to M. xenopi sequevar C (Fig. 2) (Roth et al., 1998). The ITS sequences of strains E43 and E347T were identical, but unique. They showed the closest similarity to M. xenopi, despite the fact that there was a more than 30 nucleotide difference in size and only 80% similarity to any known M. xenopi sequevar (Fig. 2). The genetic evidence revealed by 16S rDNA and ITS region sequencing indicates that these two isolates belong to a previously unclassified species.

**Growth temperature ranges and biochemical test results**

All three environmental strains, E43, E341 and E347T, were originally isolated at 42 °C. In subculture, they grew at 36–50 °C, but not at 30 °C; this was also the case for the M. xenopi ATCC strains, as well as the clinical and veterinary M. xenopi strains. At 50 °C, strain E341 grew well, whereas growth of E347T and E43 was dysgonic (Table 2). Strains E43 and E347T preferred egg media to Middlebrook. Subculture on Middlebrook 7H11 agar yielded barely visible colonies after 6 weeks incubation. Both E43 and E347T grew best on glycerol egg medium, pH 6–7. Strains E43 and E347T were initially isolated on glycerol egg medium (pH 5.5) and on pyruvate egg medium (pH 5.5), respectively, even though these media were poor supporters of growth. All strains were positive for 10-d arylsulfatase and pyrazinamidase, and negative for urease, nitrate reduction and Tween 80 hydrolysis. In the 3-d arylsulfatase test, strains E43 and E347T were negative, in contrast to the positive results obtained with all M. xenopi strains examined in this study. Strain E341 was positive for heat-stable catalase, in contrast to strains E43 and E347 which were negative. All of the environmental strains were negative for semi-quantitative catalase, β-galactosidase, acid phosphatase and 5% NaCl tolerance.

**Environmental data**

The sampling site of the environmental, genuine M. xenopi isolate (E341) was located approximately 100 km east from the coast of the Gulf of Bothnia in a rural region without any adjacent population, meaning that contamination by domestic animals or humans is unlikely. The area is rich in peatland. This was reflected by the quality of the stream water which was slightly acidic (pH 5.4) and had a high content of organic matter, as indicated by the colour value (360 mg Pt l⁻¹) and chemical oxygen demand (94 mg KMnO₄ l⁻¹). The total viable count of mycobacteria was 730 c.f.u. l⁻¹.

Strains E43 and E347T were isolated from the same geographic district as strain E341; the distance between these two sampling sites was 40 km. Strains E43 and E347T were isolated from the same stream water sample, but by different decontamination methods (Ivanainen et al., 1993). In this stream water, the total viable count of mycobacteria was high (1200 c.f.u. l⁻¹). The drainage area of the stream contained 40% peatland. The content of organic matter in the water was high (colour, 280 mg Pt l⁻¹; chemical oxygen demand, 84 mg KMnO₄ l⁻¹), and the water was acidic (pH 4.7) and exhibited a very poor buffering capacity (alkalinity, 0.09 mmol HCO₃⁻ l⁻¹).
Table 2. Growth and biochemical characteristics of environmental *M. xenopi* and *M. xenopi*-like strains assigned as *M. botniense* sp. nov.

<table>
<thead>
<tr>
<th>Test</th>
<th><em>M. xenopi</em> E341</th>
<th><em>M. botniense</em> E347&lt;sup&gt;T&lt;/sup&gt;</th>
<th><em>M. botniense</em> E43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 42 °C on:</td>
<td>+</td>
<td>-&lt;sup&gt;+&lt;/sup&gt;</td>
<td>-&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middlebrook 7H11</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol egg, pH 6.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol egg, pH 5.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvate egg, pH 6.5</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pyruvate egg, pH 5.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Middlebrook 7H11 supplemented with haemin</td>
<td>+</td>
<td>-&lt;sup&gt;+&lt;/sup&gt;</td>
<td>-&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Growth at 30 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Growth at 50 °C</td>
<td>+</td>
<td>+†</td>
<td>+</td>
</tr>
<tr>
<td>3-d arylsulfatase</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10-d arylsulfatase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyrazinamidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Catalase (&gt;45 mm foam; semi-quantitative)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Catalase (heat-stable)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Acid phosphatase</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Urease</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Nitrate reduction</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Tween 80 hydrolysis</td>
<td>-</td>
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</table>

* Growth only observed from high inocula as dysgonic colonies.
† Growth visible after 8 weeks incubation.

**DISCUSSION**

The results of 16S rDNA and ITS region sequencing, combined with those of phenotypic analyses, showed that the three environmental strains examined could be divided into two entities, one differing from earlier known species (strains E43 and E347<sup>T</sup>) and the other identical to *M. xenopi sensu stricto*. Thus, it is proposed that strains E43 and E347<sup>T</sup> are assigned to a new species, *M. botniense* sp. nov.

Strains E43 and E347<sup>T</sup> closely resembled *M. xenopi* in lipid analyses by GLC and HPLC, and biochemical testing. However, 16S rDNA and ITS sequence analyses of these strains highlighted differences which distinguished them from all other mycobacteria previously described. Moreover, a detailed GLC-MS analysis revealed the presence of two new marker fatty acids (2,4,6,8-tetramethyl-20:0 and 2,4,6,9,11-penta-22:0) in the profiles of both strains. As far as we are aware, *M. botniense* sp. nov. has not been isolated from patients. This implies that the proposed species is saprophytic, or that it has been misidentified as *M. xenopi*. Since the growth characteristics, biochemical properties and fatty acid profiles of *M. xenopi* and *M. botniense* are very similar, their separation may be difficult in a routine diagnostic laboratory. However, 16S rDNA sequencing and GLC-MS can differentiate the two species. Characteristics of *M. botniense* that were useful for distinguishing it from *M. xenopi* included dysgonic growth, especially on Middlebrook 7H11 agar, and a combination of negative results in the heat-stable catalase test and negative results in the 3-d arylsulfatase test.

HPLC analysis showed that both *M. xenopi* and *M. botniense* present two clusters of peaks characterized by their almost overlapping retention times. In genuine *M. xenopi*, the peaks of the first cluster are consistently higher (approximately double) than those of the second. In *M. botniense*, the peak heights in the two clusters are about equal.

In addition to differences in relative amounts of fatty acid and alcohol markers, minor variations may occur in the fatty acid composition within a particular mycobacterial species. As can be seen from the previously published GLC profiles (Butler *et al.*, 1993; Chou *et al.*, 1996; Jantzen *et al.*, 1989; Larsson *et al.*, 1989; Luquin *et al.*, 1989), dodecanoic acid (12:0) may or may not be detected in *M. xenopi*. Among our clinical isolates, only one of the 25 strains analysed, *M. xenopi* strain 41334, did not contain dodecanoic acid.

*M. xenopi* is a potentially pathogenic mycobacterium which is frequently detected in clinical samples in some parts of the world but, in some geographic areas, it is most uncommon (Elhelou *et al.*, 1997; Falkinham, 1996; Wolinsky, 1992; Yates *et al.*, 1997). In Finland, the recovery of *M. xenopi* from clinical samples is rare.
(less than 0.5% among annual isolates of mycobacteria other than tuberculosis cases) (National Public Health Institute, 1998). *M. xenopi* is also uncommon as a veterinary pathogen. In addition to its initial isolation from a laboratory toad by Schwabacher (1959), it has been isolated from a cat (Tomasovic et al., 1981; Jarnagin et al., 1971; Thoen et al., 1975). In experimental studies, it has been found to be pathogenic for rabbits (Demoulin-Brahy, 1978).

All mycobacteria, except for *M. tuberculosis*, appear to have reservoirs in the environment (Falkinham, 1996; Portaels, 1995). The same has been believed to apply to *M. xenopi*. To our present knowledge, however, this is the first report of isolation of *M. xenopi* from natural environments, although it has been isolated from man-made environments, e.g. tap waters, especially hot water systems (Bullin et al., 1970; Costrini et al., 1981; Kaustová et al., 1993; McSwiggan & Collins, 1974; Sniadack et al., 1993; Wright et al., 1985), and sewage (Szabó et al., 1982). The sampling site of the environmental *M. xenopi* isolate was not downstream of any recognizable source of human or animal contamination. The same water also harboured isolates of the *M. avium* complex and *M. malmoense* (Iivanainen et al., 1994; Katila et al., 1995). Peatlands and high organic matter content are apparently important to many potentially pathogenic mycobacteria in the natural environment (Iivanainen et al., 1993, 1999). In addition to this novel mycobacterial species, *M. botniense* sp. nov., several other unidentified mycobacterial groups have been isolated from stream waters, but their taxonomic status and pathogenicity are still unclear (P. Torkko, E. Iivanainen & M.-L. Katila, unpublished results).

**Separation of *M. botniense* from other slow-growing pigmented mycobacteria**

If conventional identification systems utilizing only biochemical tests are used for identification, it could be possible to misidentify *M. botniense* sp. nov. as a strain of the yellow-pigmented *M. avium* complex or *M. celatum*. GLC can be used to differentiate *M. botniense* from the other pigmented slow-growing mycobacteria, including the *M. avium* complex, but *M. botniense* is easily mistaken for *M. xenopi*. One differentiating characteristic is the lack of dodecanoic acid. Negative results in 3-d arylsulfatase and heat-stable catalase tests are also useful as confirmatory tests. If GLC-MS analysis is carried out, methyl-branched MACPs (2,4,6,x-tetramethyl-20:0 and 2,4,6,x,x-pentamethyl-22:0) can be used marker substances. The most reliable identification is achieved by 16S rDNA sequencing. Even though 2-docosanol has been regarded as a specific marker for *M. xenopi* and *M. shimoidei* (Brander et al., 1992), and now also for *M. botniense*, studies have indicated that minor amounts of 2-docosanol can be detected in *M. celatum* (Butler et al., 1993). The GLC profile of *M. celatum* resembles that of *M. xenopi* and *M. botniense*. In distinguishing *M. xenopi* and *M. botniense* from *M. celatum*, the ratio of 2-docosanol to 2-eicosanol in *M. xenopi*, or the ratio of 2-docosanol plus 2,4,6,x-tetramethyl-20:0 to 2-eicosanol in *M. botniense*, should be greater than one.

**Description of *Mycobacterium botniense* sp. nov.**

*Mycobacterium botniense* (bot.ni.en.se. L. gen. botniense of Botnia, referring to the Latin name of the province of Finland from which the isolation was made).

Colonies on egg media and on Middlebrook 7H11 agar are small, dasygonic and scotochromogenic, and produce yellow pigment. Visible growth from diluted inocula requires 5–8 weeks. Growth occurs at 37–50°C. The type strain is positive for 10-d arylsulfatase and pyrazinamidase, and negative for 3-d arylsulfatase, urease, nitrate reductase, semi-quantitative catalase, heat-stable catalase, acid phosphatase, β-galactosidase and 5% NaCl tolerance. TWEEN 80 is not hydrolysed in 10 d. In GLC-MS analysis, methyl-branched MACPs (2,4,6,x-tetramethyl-20:0 and 2,4,6,x,x-pentamethyl-22:0) are unique markers of *M. botniense*. A phylogenetic tree based on the evaluation of 16S rDNA sequences places *M. botniense* sp. nov. among the slow-growing mycobacteria, close to *M. xenopi*. The strains have been submitted to the American Type Culture Collection as ATCC 700701E (= E3477) and ATCC 700702 (= E43).

**ACKNOWLEDGEMENTS**

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Mycobacterium xenopi and Mycobacterium botniense sp. nov.


